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ENCAPSULATION OF FISH OIL IN NANOFIBERS BY EMULSION ELECTROSPINNING: PHYSICAL CHARACTERIZATION AND OXIDATIVE STABILITY

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ABSTRACT

The encapsulation of fish oil in poly(vinyl alcohol) (PVA) nanofibers by emulsion electrospinning was investigated. Independently of the emulsifier used, whey protein isolate (WPI) or fish protein hydrolysate (FPH), PVA concentration had a high influence on fiber morphology. Fibers without bead defects were only produced for solutions with 10.5% (w/w) PVA, which presented sufficient number of polymer chain entanglements. On the other hand, increasing oil load from 1.5 to 3% (w/w) resulted in fibers with larger diameters containing spindle-like enlargements interspersed. High omega-3 encapsulation efficiency ($92.4 \pm 2.3\%$) was obtained for fibers produced from 10.5% (w/w) PVA-5% (w/w) emulsion blend stabilized with WPI, resulting in an oil load capacity of $11.3 \pm 0.3\%$. Moreover, the encapsulated oil was randomly distributed as small droplets inside the fibers. However, the electrospun fibers presented a higher content of hydroperoxides and secondary oxidation products (e.g. 1-penten-3-ol, hexanal, octanal and nonanal) compared to emulsified and unprotected fish oil.

Keywords: fish oil, nanofibers, emulsion, electrospinning, oxidative stability

1. INTRODUCTION

Omega-3 polyunsaturated fatty acids (PUFA), particularly eicosapentaenoic (C20:5n-3, EPA) and docosahexaenoic (C22:6n-3, DHA), have numerous beneficial health effects such as prevention of

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cardiovascular disease, improvement of the anti-inflammatory response and development of brain and eye retina in infants (Uauy & Valenzuela, 2000; Ward & Singh, 2005). These PUFA need to be ingested through the diet since humans have a low conversion rate of the essential α -linolenic fatty acid (ALA) to EPA and DHA (Colussi et al., 2007). As a consequence, the food industry has an increasing interest in developing omega-3 enriched foods. Accordingly, efficient strategies for protecting these highly unsaturated fatty acids against oxidation when incorporated into foods are necessary in order to avoid both the loss of nutritional value and the formation of unpleasant off-flavors. For that purpose, omega-3 delivery systems (e.g. fish oil-in-water emulsions and microencapsulated fish oil) are often employed since they can protect the oil against prooxidants in the food system by providing a physical barrier between the omega-3 oil and oxygen or prooxidants (e.g. metal ions) (Jacobsen & Nielsen, 2007). The employment of emulsified omega-3 oils is more suitable for liquid or semi-liquid foods due to handling/mixing issues. Although a higher oxidative stability was obtained when adding fish oil emulsions to milk, negative results were obtained when adding emulsified fish oil to other food systems such as yoghurt and salad dressings (Let et al., 2007). On the other hand, microencapsulated fish oil, commonly produced by spray-drying, presents in many cases a higher oxidative stability when compared to neat fish oil (Nielsen & Jacobsen, 2013). Nevertheless, the application of the resulting microcapsules is limited to dry food products (e.g. powdered infant formula) due to their poor solubility (Drusch, 2012). Furthermore, the spray-drying process requires air at high temperature (170-190 °C) which causes initial oxidation of the oil (Serfert et al., 2009). In addition, powdered product is deposited in a large amount on the outlet pipe and chamber wall during spray-drying, which reduces yield (Wan et al., 2011).

Therefore, the development of alternative omega-3 PUFA delivery systems, which are easy to disperse and which lead to improved oxidative stability of omega-3 enriched food products are required. In this sense, electrospinning processing is a straightforward and versatile encapsulation technique suitable for the production of nano-microfibers containing bioactive compounds (Aceituno-Medina et al., 2013; Stephansen et al., 2014). The process uses a high-voltage electro-static field to charge the surface of a polymer solution droplet formed at the end of a capillary tube. Mutual charge repulsion

causes a force directly opposite to the surface tension which elongates the droplet forming a conical shape known as the Taylor cone. When the electrical forces overcome the surface tension, an electrically charged jet is ejected from the tip of the Taylor cone. On the way to the collector, the jet is stretched out due to several instabilities (e.g. whipping or bending motions) which favor the evaporation of the solvent resulting in dried fibers (Doshi & Reneker, 1995; Frenot & Chronakis, 2003). Electrospinning processing presents several advantages such as: i) it does not require heat, thus avoiding deterioration of the active compound, and ii) it results in decreased encapsulates size which allows their incorporation into food systems without affecting the sensory qualities of the product (Li et al., 2013; Weiss et al., 2012).

Recently, lipophilic compounds such as β -carotene and fish oil have successfully been entrapped into nanofibers produced by electrospinning of zein aqueous-ethanol solutions (Fernandez et al., 2009; Moomand & Lim, 2014). However, zein, which is a hydrophobic food-approved biopolymer, has a high cost which reduces its use in food applications (Kushwaha & Kawtikwar, 2013). In this context, emulsion electrospinning is a promising alternative since it allows for the encapsulation of hydrophobic compounds in low-cost hydrophilic polymers, avoiding also the use of organic solvents which are restricted in food systems (Arecchi et al., 2010). A few studies have already been reported on the encapsulation of lipophilic compounds (e.g. mineral oil, hexadecane, limonene, retinyl palmitate and n-butyl acetate) using this technique (Angeles et al., 2008; Arecchi, et al., 2010; Camerlo et al., 2014; Gordon et al., 2015). These authors employed small-sized molecular surfactants such as Pluronic P105 (Angeles, et al., 2008), Tween 20 (Arecchi et al., 2010; Camerlo et al., 2014) and Brij O10, and mixtures of Tween 80 and Span 80 (Gordon et al., 2015) as emulsifiers. Apart from the study of Angeles et al. (2008) which used polyethylene oxide (PEO), the remaining studies employed poly(vinyl) alcohol (PVA) as polymer since it has a higher thermal stability and lower cost than PEO, it is biocompatible, non-toxic, and presents sufficient amounts of chain entanglements which allow for the formation of fibers by electrospinning (Weiss et al., 2012). To the best of the authors' knowledge, no research work on the encapsulation of fish oil in nanofibers obtained by emulsion electrospinning has been previously published.

Thus, the aim of this work was to study the development of omega-3 delivery systems by emulsion electrospinning using PVA as polymer. Firstly, the effect of polymer concentration, oil load and type of emulsifier on the morphology of the fibers was evaluated. Secondly, the oxidative stability during storage of fish oil encapsulated in selected fibers was studied. Contrarily to previous works, which used low molecular weight surfactants, proteins such as whey protein isolate (WPI) and fish protein hydrolysate (FPH) were assayed as emulsifiers in this study. WPI is normally used in the food industry to stabilize oil-in-water emulsions because of its functional, bioactive and nutritional properties (Adjonu et al., 2014). Recently, FPH has also been reported as a promising alternative emulsifier leading to physical and oxidative stable fish oil-in-water emulsions (García-Moreno et al., 2016; Morales-Medina et al., 2016).

2. MATERIALS AND METHODS

2.1 Materials

Commercial cod liver oil was kindly provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway) and stored at -40 °C until use. The fatty acid composition of the fish oil was determined by fatty acid methylation (AOCS, 1998a) followed by separation through GC (AOCS, 1998b). It was as follows (% , w/w): 9.5% palmitic acid (C16:0), 8.7% palmitoleic acid (C16:1n-7), 2.0% stearic acid (C18:0), 16.3% oleic acid (C18:1n-9), 4.8% vaccenic acid (C18:1n-7), 1.8% linoleic acid (C18:2n-6), 2.6% α -linolenic acid (C18:3n-3), 12.6% gadoleic acid (C20:1n-11), 9.2% eicosapentaenoic acid (C20:5n-3), 6.0% cetoleic acid (C22:1n-11), 11.4% docosahexaenoic acid (C22:6n-3) and 15.1% others. The tocopherol content of the fish oil was: α -tocopherol, 200 \pm 3 μ g/g oil; β -tocopherol, 5 \pm 1 μ g/g oil; γ -tocopherol, 96 \pm 3 μ g/g oil and δ -tocopherol, 47 \pm 1 μ g/g oil. The peroxide value (PV) of the fish oil used was 0.38 \pm 0.04 meq/kg oil. Whey protein isolate (WPI), with commercial name Laprodan® DI-9224, was kindly donated by ARLA (ARLA Food Ingredients, Viby, Denmark). Fish protein hydrolysate (FPH) with a degree of hydrolysis of 4% was produced from sardine muscle using Alcalase 2.4 L (Novozymes, Bagsværd, Denmark) as described by García-Moreno et al. (2016). Three different types of poly(vinyl alcohol) (PVA) were purchased from Sigma-Aldrich (Brøndby,

Denmark): a) Mowiol® 4-98 (molecular weight = 27,000 Da; degree of hydrolysis = 98-98.8%), b) Mowiol® 8-88 (molecular weight = 67,000 Da; degree of hydrolysis = 86.7-88.7%), and c) Mowiol® 18-88 (molecular weight = 130,000 Da; degree of hydrolysis = 86.7-88.7%). PVA partially saponified (130,000 Da) was kindly provided by Kuraray Europe GmbH.

2.2 Preparation of fish oil-in-water emulsion

Six different emulsions, stabilized with WPI or FPH and with fish oil content of 5, 7.5 and 10% (w/w), were produced. Aqueous phases were prepared by dissolving WPI (1%, w/w) or FPH (2%, w/w) in distilled water and the pH was adjusted to pH 2 by addition of 0.1 N HCl. Acidic pH (pH 2) led to the lowest interfacial tension for sardine hydrolysate, which may be related with increased solubility of fish protein at low pH (Morales-Medina et al., 2016). The aqueous solutions were then stirred overnight at 5 °C to allow complete rehydration of the protein. Primary homogenization was done by adding the fish oil (5, 7.5 and 10%, w/w) slowly to the aqueous phase during mixing at 16,000 rpm (Ystral mixer, Ballrechten-Dottingen, Germany). The fish oil was added during the first minute of mixing, and the total mixing time was 3 min. Secondary homogenization was done on a microfluidizer (M110L Microfluidics, Newton, MA, USA) equipped with a ceramic interaction chamber (CIXC, F20Y, internal dimension 75µm). Emulsions were homogenized at a pressure of 9,000 psi, running 3 passes. Another emulsion (12% fish oil-in-water emulsion, w/w) stabilized with WPI was also produced only for measurement of lipid oxidation.

2.3 Preparation of fish oil-in-water emulsion-PVA solutions for electrospinning

Preliminary experiments (data not shown) indicated that adding PVA to the aqueous phase before the homogenization process led to the formation of more and larger beads in the fibers. This may be associated to a reduction in the viscosity of the solution as consequence of the homogenization process. Furthermore, adding PVA before the homogenization process also resulted in larger emulsion droplet size, which may decrease the encapsulation efficiency. The influence of the molecular weight of PVA (27, 67 and 130 kDa) on fiber morphology was also tested in preliminary studies (data not shown). For 10% (w/w) PVA solutions, a stable Taylor cone could not be obtained for 27 kDa PVA

even when using a low flow rate (0.01 mL/min). PVA of 67 and 130 kDa led to stable Taylor cones, however fibers with more and larger beads were produced when using PVA 67 kDa when compared to PVA 130 kDa (data not shown).

Therefore, PVA 130 kDa was employed in this study and was added to the aqueous phase after the homogenization process as follows. PVA (15%, w/w) and acetic acid (1%, w/w) were dissolved in distilled water (adjusted at pH 2 by addition of 0.1 N HCl) at 80 °C under constant stirring for 3 h. The solution was allowed to cool to room temperature under stirring for 2 h and then overnight without stirring. This PVA polymer solution was blended with fish oil-in-water emulsions in order to obtain electrospinning solutions with PVA concentration of 7.5, 9 and 10.5% (w/w). The mixing process was carried out under nitrogen atmosphere by using magnetic stirring for 2 h at 5 °C in the dark. A full factorial experimental design with 3 factors (type of emulsifier: WPI and FPH; oil load in emulsions: 5, 7.5 and 10% w/w; and PVA concentration: 7.5, 9 and 10.5% w/w) and no replicates was carried out. This led to 18 different solutions for electrospinning (Table 1). Samples were used immediately after production for electrospinning processing and subsequently stored at 5 °C in the dark until further analysis.

2.4 Characterization of solutions for electrospinning

2.4.1 Droplet size

Droplet sizes were measured by laser diffraction in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK). The PVA-emulsion blends were diluted in recirculating water (3000 rpm), until it reached an obscuration of 12%. The refractive indices of sunflower oil (1.469) and water (1.330) were used as particle and dispersant, respectively. Results are given in surface mean diameter ($D_{3,2}$). Measurements were made in duplicate at day 1, 3 and 7 after production. Droplet size of the emulsion was also measured immediately after production to evaluate the effect of adding PVA.

2.4.2 Viscosity

The viscosity of electrospinning solutions was measured using a stress controlled rheometer Stresstech (Reologica Instruments AB, Lund, Sweden) equipped with a CC25 standard bob cup system in a

temperature vessel. Measurements were done at 20°C over a shear stress range from 0.0025 to 20 Pa. Viscosity was measured twice on each solution at day 1 after production and it was expressed in mPa·s.

2.5 Electrospinning process

The electrospinning solutions were added to a syringe and placed in a syringe pump (New Era Pump Systems, Inc., USA). A 16 G needle (Proto Advantage, Canada) was used. The syringe pump delivered the PVA-emulsion blends with a flow rate of 0.02 ml/min. Using a high voltage power supply (Gamma High Voltage Research, USA), an electric field of 20 kV was applied between the spinneret of the syringe and a 5 × 5 cm collector plate made of stainless steel with alumina foil wrapped around it. The distance between the syringe tip and the collector plate was 10 cm. The electrospinning process was conducted at room temperature. From the 18 electrospinning solutions shown in Table 1, electrospun mats were obtained with a fish oil content ranging from 12.0 to 38.4% (w/w). Fibers with a final oil content of 12.2% (w/w) containing 5% (w/w) fish oil-in-water emulsions stabilized with WPI were produced in large quantity for peroxide value and secondary oxidation products determinations. For that purpose, fibers were produced in batches of 5 h. Fibers with 12.2% (w/w) oil containing 5% (w/w) fish oil-in-water emulsions stabilized with WPI were also produced for peroxide value determination from: i) a solution containing 13.5% (w/w) PVA, and ii) a solution containing 10.5% (w/w) PVA and 100 ppm of ethylenediaminetetraacetic acid (EDTA) in the parent emulsion.

2.6 Characterization of fibers

Immediately after production, fibers were stored in a desiccator until analyses.

2.6.1 Morphology

The morphology of the fibers was investigated using scanning electron microscopy (SEM) (FEI Inspect, Hillsboro, OR, USA). Approximately 0.5×0.5 cm of the fiber sheet was placed on carbon tape and sputter coated with gold, 10 s, 40 mA utilizing a Cressington 208HR Sputter Coater (Cressington Scientific Instruments, Watford, England). The mean fiber or bead diameters were determined from

the SEM images by image analysis (ImageJ, National Institutes of Health). To this purpose, the diameter of 100 randomly selected fibers and all the beads (if any) presented in the micrograph were measured using the ImageJ software (National Institutes of Health).

2.6.2 Lipid distribution

Before mixing with the PVA solution, selected emulsions were stained with Nile red and fluorescein isothiocyanate to visualize lipids and proteins, respectively. Then, the distribution of the fish oil within the fibers was analyzed with laser scanning confocal microscopy (LSCM) (Zeiss LSM 780, Jena, Germany). The fibers were placed between a coverslip and a glass slide, immersed in immersion oil, and fluorescence after irradiation at 580 nm or 488 nm was analyzed. The images were processed using ZEN 2012 lite software (Zeiss, Jena, Germany). Moreover, the droplet size distribution of the emulsion-loaded electrospun fibers after re-dispersion in distilled water was measured as previously described in section 2.4.2. For that purpose, 150 mg of fibers were dissolved in 15 mL of distilled water at room temperature under magnetic stirring for 30 min. The resulting dispersion was filtered (pore size: 150 nm) in order to remove the possible rest of fibers.

2.6.3 Encapsulation efficiency and loading capacity

Encapsulation efficiency (EE) and loading capacity (LC) were determined by measuring the non-entrapped fish oil according to Moomand and Lim (2014) with some modifications. Electrospun fibers (50 mg) were submerged in heptane (10 mL) and gently shaken (100 rpm) for 15 min. The mixture was filtered and the absorbance of the liquid was measured at 250 nm (UV-1800, Shimadzu, Japan). The amount of oil present in the liquid was determined from a calibration curve ($R^2=0.99$), prepared by dissolving various quantities of fish oil in heptane. The EE and LC values were calculated as:

$$EE (\%) = \frac{A-B}{A} * 100 \quad [1]$$

$$LC (\%) = \frac{A-B}{C} * 100 \quad [2]$$

where A is the total theoretical amount of fish oil, B is the free amount of fish oil in the collection solution, and C is the weight of the fibers. Measurements were carried out in triplicate.

2.6.4 Oxidative stability

For lipid oxidation measurements, immediately after production, fibers were stored in 100 mL brown bottles at 40 °C in the dark for 14 days. For peroxide value determination each bottle contained 400 mg fibers, and for secondary oxidation products, the bottles contained 150 mg fibers. Unprotected and emulsified fish oil (12% fish oil-in-water emulsion, w/w; stabilized with WPI) was also analyzed. Samples were taken at day 0, 4, 9 and 14 for analysis.

2.6.4.1 Determination of peroxide value

Lipids were extracted from fibers according to the Bligh and Dyer method using a reduced amount of the chloroform/methanol (1:1, w/w) solvent (Bligh & Dyer, 1959). Two extractions were made from each sample. Peroxide value (PV) was determined on lipid extracts using the colorimetric ferric-thiocyanate method at 500 nm as described by Shantha and Decker (1994).

2.6.4.2 Secondary oxidation products – Dynamic headspace GC-MS

Approximately 40 mg of fibers and 30 mg internal standard (4-methyl-1-pentanol, 30 µg/g water) were weighted out in a 100 mL purge bottle, to which 5 mL of distilled water and 1 mL antifoam (Synperonic 800 µL/L water) were added. The bottle was heated to 45°C in a water bath while purging with nitrogen (flow 340 mL/min, 30 min). Volatile secondary oxidation products were trapped on Tenax GR tubes. The volatiles were desorbed again by heating (200°C) in an Automatic Thermal Desorber (ATD-400, Perkin Elmer, Norwalk, CN), cryofocused on a cold trap (-30°C), released again (220°C), and led to a gas chromatograph (HP 5890IIA, Hewlett Packard, Palo Alto, CA, USA; Column: DB-1701, 30 m x 0.25 mm x 1.0 µm; J&W Scientific, CA, USA). The oven program had an initial temperature of 45°C for 5 min, increasing with 1.5°C/min until 55°C, with 2.5°C/min until 90°C, and with 12.0°C/min until 220°C, where the temperature was kept for 4 min. The individual compounds were analyzed by mass-spectrometry (HP 5972 mass-selective detector, Agilent Technologies, USA; electron ionization mode, 70 eV; mass to charge ratio scan between 30 and 250). Emulsified fish oil (4g, 12% oil-in-water emulsion, w/w; stabilized with WPI) and unprotected fish oil (4 g) were also analyzed following the same procedure using a nitrogen flow of 150 mL/min (30 min).

In the case of unprotected fish oil, water and antifoam were not added and the bottle was heated at 60°C. The individual compounds were identified by both MS-library searches (Wiley 138 K, John Wiley and Sons, Hewlett-Packard) and by authentic external standard. The external standards employed were 2-ethylfuran, 1-penten-3-one, pentanal, 1-penten-3-ol, (*E*)-2-pentenal, hexanal, (*E,E*)-2,4-heptadienal, octanal, nonanal and (*E,Z*)-2,6-nonadienal (Sigma-Aldrich, Brøndby, Denmark). These compounds were dissolved in 96% ethanol for fibers and emulsion samples, and rapeseed oil for fish oil samples. Then, the solutions were diluted to concentrations of approximately 1, 5, 10, 25 and 100 µg/g. Three calibration curves were made by injecting these solutions (30 µg) on 40 mg of fibers dissolved in 5 mL of water, 4 g of emulsified fish oil and 4 g of unprotected fish oil, respectively. Measurements were made in triplicates for each sample.

2.7 Statistical analysis

Statgraphics Centurion XV (Statistical Graphics Corp., Rockville, MD, USA) was used for data analysis. Data were expressed as mean \pm standard deviation with two analytical replicates. No experimental replicates were carried out. Firstly, multiple sample comparison analysis was performed to identify significant differences between samples. Secondly, mean values were compared by using the Tukey's test. Differences between means were considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Properties of emulsion-PVA blends

3.1.1 Droplet size and physical stability

The droplet size distribution, and thus the physical stability, of the electrospinning solutions are important factors to consider since they may affect the lipid distribution and entrapment of fish oil within the nanofibers. Accordingly, the effect of blending PVA solutions and fish oil in-water emulsions on droplet size distribution was evaluated for solutions 1-3 (Table 1). After blending, the droplet size distribution was similar to the original 5% (w/w) fish oil-in-water emulsion stabilized with WPI (data not shown). This is in agreement with the studies of Arecchi et al. (2010) and Gordon et al.

(2015) which indicated that adding PVA did not modify droplet size distribution of emulsions stabilized with small-sized molecular surfactants such as Tween 20 and Brij O10, respectively.

Secondly, the droplet size distribution of the emulsion-PVA blends was studied after production and during storage. Fig. 1a shows the droplet size of the emulsion-PVA (10.5%, w/w) blends after 1 day of production. Independently of the oil load in the original emulsion, the electrospinning solutions containing emulsions stabilized with WPI presented not significant differences ($p>0.05$) in their droplet size distribution, with $D_{3,2}$ values ranging from 130 ± 1 to 139 ± 8 nm. This was also observed for 9 and 7.5% (w/w) PVA-emulsion blends (data not shown). The droplet sizes of the PVA-emulsion blends were in line with previously reported results for 10% (w/w) fish oil-in-water emulsions stabilized with WPI ($D_{3,2}=129\pm1$ nm) (Horn et al., 2013). For electrospinning solutions containing emulsions stabilized with FPH, significantly ($p<0.05$) larger droplet sizes were obtained independently of the oil concentration when compared to solutions containing emulsions stabilized with WPI. Furthermore, the droplet size of these solutions increased significantly ($p<0.05$) with the oil load of the original emulsion, with $D_{3,2}$ values varying from 137 ± 1 to 187 ± 1 nm for solutions containing 5 and 10% (w/w) fish oil-in-water emulsions, respectively. This indicates a lower adsorption capacity of FPH at the interface, which only allows the stabilization of larger droplets with a reduced interfacial area. Similar results were found for all PVA concentrations (data not shown).

Contrary to parent emulsions, which slightly destabilized during storage, electrospinning solutions containing emulsions (5, 7.5 and 10% oil, w/w) stabilized with WPI did not differ significantly ($p>0.05$) in their surface mean diameters after one week of storage (Fig 1b). Similar observations were made for emulsions dispersed in PVA solutions with low concentrations (9 and 7.5%, w/w), and it may be attributed to a stabilizing effect of PVA on the emulsions, which reduces flocculation and coalescence by acting as emulsifier (Ajalloueian et al., 2014) and/or by increasing solution viscosity. Similarly, PVA solutions containing emulsions (5 and 7.5%, w/w) stabilized with FPH were also physically stable during one week (Fig. 1c). However, despite the stabilizing effect of PVA, the PVA-emulsion (10%, w/w; FPH) blend destabilized after 3 days of storage (Fig. 1c). This is explained by

the high physical instability of the original emulsion, which is a consequence of the reduced emulsifying capacity of FPH.

3.1.2 Viscosity

Different factors have been reported to affect fiber formation and morphology; including solution properties (e.g. viscosity, electrical conductivity, surface tension and solvent volatility), environmental conditions (e.g. temperature and humidity), and process parameters (voltage, flow rate and spinning distance) (Chronakis, 2010; Ramakrishna, 2005). Solution viscosity is mainly dependent on the solvent and on polymer molecular weight and concentration (Weiss et al., 2012). It is a measure for the chain entanglements in the solution, for which reason a minimum viscosity is required to stabilize the jet during the process and to produce continuous fibers (Ghorani & Tucker, 2015). Fig. 1d shows the dynamic viscosity values for the PVA-emulsion blends. All solutions presented Newtonian behavior with viscosity values ranging from 138 to 608 mPa·s. These values are considerably higher compared to the original emulsions (<5 mPa·s) due to polymer-polymer and polymer-solvent interactions (Arecchi et al., 2010). In this study, the PVA concentration was the main factor affecting the viscosity of the PVA-emulsion blends. The viscosity increased when raising the polymer concentration, however increasing fish oil load also slightly increased the viscosity of PVA-emulsion blends. These findings are in agreement with the results reported by Arecchi et al. (2010) and Camerlo et al. (2014) on PVA solutions containing limonene and n-hexadecane emulsions, respectively. In the same line, Moomand and Lim (2015) also found a rise in the viscosity of zein aqueous-alcohol solutions when increasing fish oil load from 17 to 30% (w/w). On the other hand, it should be noted that the type of emulsifier employed (WPI or FPH) had a minor influence on viscosity (Fig. 1d).

3.2 Fibers morphology

Uniform electrospun fibers with a circular cross-section and void of defects (e.g. beads) were obtained when using 10.5% (w/w) PVA-emulsion blends independently of the emulsifier employed. Fibers having a similar average diameters were obtained from 10.5% (w/w) PVA-5% (w/w) emulsion blends stabilized with WPI or FPH; 172 ± 44 and 163 ± 45 nm respectively (Fig. 2a and 3a). These results are in

line with previously reported studies of PVA fibers; 171 ± 61 nm for 8% (w/w) PVA (130 kDa) (Arecchi et al., 2010) and 221 ± 34 nm for 8% (w/w) PVA (205 kDa) (Gordon et al., 2014). According to Tao and Shivkumar (2007), the average fiber diameter increased with concentration and molecular weight of PVA. Nevertheless, Arecchi et al. (2010) obtained fibers with similar average diameter for the same type of PVA but using lower concentrations. This may be due to the acidic pH used in this study (pH 2) which led to a decrease in solution viscosity as consequence of the disentanglement of polymer chains (Rwei & Huang, 2009).

Next, the effect of oil load on fiber morphology for the 10.5% (w/w) PVA-emulsion blends was investigated. For solutions stabilized with WPI, increasing the oil load from 1.5 to 3% (w/w) did not affect fiber formation (Fig. 2). However, fibers obtained from solutions containing a higher oil concentration had larger and more polydispersed diameters, ranging from 172 ± 44 to 252 ± 68 nm (Fig. 2). Similar results were obtained by Gordon et al. (2014) for PVA fibers containing retinyl palmitate in isohexadecane (oil phase), with fiber diameters of 246 ± 71 and 268 ± 80 nm for 5.8 and 15% (w/w) oil phase, respectively. It should also be noted that fibers produced from solutions with an oil load higher than 1.5% (w/w), showed spindle-like enlargements interspersed along the fibers (Fig. 2b-c). These morphological changes are different from the bead defects observed by Arecchi et al. (2010) when increasing n-hexadecane content in the PVA-emulsion blend from 0.5 to 1 and 1.5% (w/w). It may be explained by the higher PVA concentration employed in this study (10.5%, w/w), which leads to a higher elongation viscosity and reduced surface tension (Rwei & Huang, 2009). There was no difference between using WPI or FPH as emulsifier in the 10.5% (w/w) PVA-emulsion blends (data not shown).

Independently of the emulsifier employed, the PVA concentration had a major impact on fiber morphology. SEM micrographs of electrospun mats obtained from PVA-5% (w/w) emulsion blends stabilized with FPH are depicted in Fig. 3, which shows that decreasing PVA concentration from 10.5 to 9% (w/w) led to the formation of elongated spindle-like beads with an average diameter of 295 ± 42 nm (Fig. 3b). For 7.5% (w/w) PVA, beads-on-string morphology was observed, with the beads being

more spherical and with an increased average diameter of 784 ± 422 nm (Fig. 3c). Reducing the polymer concentration led to fewer chain entanglements, which decreased the viscoelasticity of the jet, thus causing formation of beads since the Rayleigh instability could not be completely avoided (Chang et al., 1999). Although it was expected that increasing the fish oil load (1.5-2.5%, w/w) would increase the average fiber diameter, the mean diameter of the fibers decreased from 163 ± 45 to 120 ± 39 and 120 ± 59 nm for fibers obtained from solutions containing 10.5, 9 and 7.5 PVA% (w/w), respectively (Fig. 3). Even though it is well known that decreasing PVA concentration leads to a decreased fiber diameter (Koski et al., 2004; Rwei & Huang, 2009), this decrease may be also attributable to the concentration of flocculated or coalesced droplets of fish oil in the spindle-like or bead structures. A similar finding was reported by Arecchi et al. (2010) when increasing n-hexadecane load in 8% (w/w) PVA fibers.

3.3 Characterization of selected fibers

Fibers made from the 10.5% (w/w) PVA-emulsion blend and loaded with 1.5% (w/w) fish oil stabilized with WPI (Exp. 1 in Table 1) were selected for further characterization. This solution was chosen since: i) 10.5% (w/w) PVA was required to obtain uniform fibers without defects, and ii) it presented the lowest droplet size and it was physically stable during storage, which may benefit the entrapment of fish oil inside the fibers.

3.3.1 Lipid encapsulation and distribution

Electrohydrodynamic processing offers the possibility to obtain nano-microstructures loaded with large amounts of active compounds. High EE is of outmost significance when working with bioactive compounds prone to oxidation (e.g. fish oil), since it can minimize the exposure of free bioactive compounds to oxygen (Torres-Giner et al., 2010). For the selected PVA-emulsion blend, the initial emulsion droplet size ($D_{3,2} = 130 \pm 1$ nm, Fig. 1a) and the average fiber diameter (172 ± 44 nm, Fig. 2a) indicated that most of the fish oil droplets might be entrapped within the PVA fiber. Indeed, this was confirmed by a high EE ($92.4 \pm 2.3\%$). This value is within the range of the results reported by Moomand and Lim (2014) for zein fibers containing 30% fish oil ($91.19 \pm 1.09\%$) and by Drusch et al.

(2012) for fish oil microencapsulates produced with casein hydrolysates using spray-drying (92.4%). Taking into account that the theoretical fish oil content in the fibers was 12.2% (w/w), the trapping efficiency achieved led to a LC of $11.3 \pm 0.3\%$.

Another important factor affecting the fish oil oxidative stability is its distribution within the fibers (Moomand & Lim, 2014). Fig. 4a shows the confocal image of the fibers. Both small and larger green ellipses, marking the protein, were randomly dispersed along the fibers. The small green ellipses could be due to WPI covering the fish oil droplets, whereas the larger green ellipses may mark free WPI that was not adsorbed at the interface in the parent emulsion. The change from spherical to elliptical shape of the droplets was caused by a stretching in the axial direction during the electrospinning process (Gordon et al., 2015). Fish oil (marked in red) was also dispersed along the fibers. However, from the confocal image it was difficult to determine if the fish oil was dispersed as droplets or in a continuous core. To further evaluate the distribution of the oil inside the fibers, the oil-loaded fibers were re-dispersed in distilled water. Fig. 4b depicts the droplet size distribution of the re-dispersed fibers and of the electrospinning solution, which were both almost identical to the parent emulsion, indicating that most of the fish oil was distributed as droplets (with similar size as in the initial emulsion) inside the fibers. This is in accordance with the results of Gordon et al. (2015) on fibers containing retinyl pamate, and it is also in agreement with the study of Angeles et al. (2008) which reported that the final particle size incorporate into the fibers was highly influenced by the initial emulsion droplet size. However, Fig. 4b also shows a small peak around $100 \mu\text{m}$ for the re-dispersed fibers. It may be the result of agglomeration of some large droplets in the core of the fibers forming a “core-sheath” structure, favored by the evaporation and jet stretching process during electrospinning (Xu et al., 2008; Yarin, 2011).

3.3.2 Oxidative stability

Hydroperoxides, which are also known as primary oxidation products, are formed in the early phase of lipid oxidation. Although hydroperoxides are tasteless, they decompose in the presence of heat or metal ions into secondary volatile oxidation products (e.g. alcohols, aldehydes and ketones) which are

responsible for the off-flavours (Jacobsen et al., 2013). Table 2 shows that the PV during storage of the fibers was significantly higher ($p<0.05$) when compared to the values found for the unprotected and emulsified fish oil. Contrary to emulsified fish oil, a lag phase was not observed for the unprotected fish oil and fibers since PV increased rapidly between days 0 and 4 (especially for fibers). Considering secondary oxidation products, unprotected fish oil showed a high increase in the concentration of volatiles derived from omega-3 PUFA oxidation such as 1-penten-3-ol and (*E,E*)-2,4-heptadienal (Fig. 5a). The presence of these volatiles was also significant in the emulsified fish oil but to a lower extent compared to unprotected fish oil. On the other hand, emulsified fish oil presented a higher increase in the concentration during storage of 2-ethylfuran, another volatile derived from oxidation of omega-3 fatty acids (Fig. 5b). Nevertheless, and as observed for PV, the nanofibers presented the highest increase in the concentration of volatiles during storage. Particularly important was the formation of 1-penten-3-ol and other volatiles such as hexanal and nonanal which were derived from omega-6 and omega-9 fatty acids, respectively (Fig. 5c).

Unexpectedly, these results revealed the inability of the fibers to protect the encapsulated fish oil against oxidation, which was more oxidized than the unprotected counterparts. Even immediately after production, the fibers presented a PV of 54.6 ± 9.3 meq O_2 /kg oil (Table 2). Therefore, several strategies were carried out in an attempt to enhance the resistance to oxidation of the electrospun nanofibers. First, the electrospinning process was carried out under partial nitrogen atmosphere by using a home-made chamber in order to avoid oxidation of the untrapped oil during production of the electrospun mats. However, the nanofibers obtained under this partial inert atmosphere only showed a slightly reduced PV (Table 2). Next, the concentration of PVA in the electrospinning solution was increased to 13.5% (w/w) aiming at increasing the fiber diameter, and thus improving the entrapment of fish oil. Moomand and Lim (2014) reported that zein nanofibers loaded with fish oil were more oxidatively stable when increasing encapsulation efficiency from 91.19 ± 1.09 to $95.88\pm0.23\%$. Surprisingly, PV at day 0 for fibers produced under nitrogen from a 13.5% (w/w) PVA-emulsion blend was significantly higher ($p<0.05$) than for fibers obtained from a 10.5% (w/w) PVA-emulsion blend (Table 2). Finally, EDTA (100 ppm), which is an antioxidant with metal chelating

properties, was added to the emulsion before blending with the PVA solution. The PV at day 0 for the obtained fibers was not significantly improved (43.9 ± 7.8 meq O₂/kg oil). Moreover, PV during storage of the 10.5% (w/w) PVA-emulsion blend containing 1.5% (w/w) fish oil and EDTA was considerably higher when compared to emulsified fish oil (Table 2). This finding may denote a negative influence of PVA as carrier matrix on the oxidative stability of encapsulated fish oil. Indeed, the prooxidant effect of PVA was confirmed by the results obtained for a PVA-emulsion blend when using PVA from a different producer (Kuraray Europe GmbH). Table 2 shows that PV of the PVA (Kuraray)-emulsion blend was significantly ($p < 0.05$) higher at days 9 and 14 when compared to emulsified fish oil. PV of PVA (Kuraray)-emulsion blend was also higher than PVA (Sigma)-emulsion blend at days 9 and 14, probably due to the chelating effect of EDTA added to the latter solution. PVA which is produced in metal equipment has been reported to contain trace quantities of metals (e.g. Ca, Fe, Al) (Zhu, Gao, Xu, & Shi, 2008). It is well known that metal-catalyzed decomposition of lipid peroxides not only generates free radicals, which initiate further oxidation reactions, but also leads to the formation of secondary volatile oxidation products (Frankel, 2005). In this sense, the interaction of PVA metal impurities with existing peroxides present in the de-emulsified fish oil forming the “core-sheath” structure may be an important driving force for lipid oxidation in the nanofibers. Contrarily to these results, Moomand and Lim (2014) and Torres-Giner et al. (2010) encapsulated fish oil in oxidatively stable zein nanofibers and nanocapsules, respectively. In the same line, de Freitas Zômpero et al. (2015) reported that zein is more adequate than PVA to encapsulate β -carotene by electrospinning since it led to higher protection of this bioactive upon exposure to ultraviolet light. These differences may be explained by the antioxidant properties of zein which are due to its content in carotenoids (e.g. β -carotene, zeaxanthin and lutein) and sulphur amino acids exhibiting radical scavenging properties (Moomand & Lim, 2014). Nevertheless, the current high-cost manufacturing methods of zein make it an uneconomical material for food applications (Kushwaha & Kawtikwar, 2013).

It should be also mentioned that the process conditions employed could have also had a negative effect on the oxidative stability of the fibers. In this sense, the long duration of the production batches

together with the no total inert atmosphere used may have favored oxidation of untrapped fish oil during fibers production.

4. CONCLUSIONS

Nanofibers loaded with fish oil can be successfully produced by emulsion electrospinning using PVA as polymer and WPI or FPH as emulsifiers. WPI exhibited a higher emulsifying property than FPH, and led to more physically stable PVA-emulsion blends with smaller droplet size. A PVA concentration of 10.5% (w/w) allowed sufficient chain polymer entanglements required in order to avoid the formation of elongated spindle-like beads or beads-on-string morphologies. Increasing oil load of the PVA-emulsion blends only had a small influence on the formation of fiber defects, apart from the appearance of spindle-like enlargements interspersed along the fibers. Although most of the fish oil was encapsulated inside the fibers as small droplets, the fibers presented a poor oxidative stability. This may be related to the presence of traces of metals (e.g. Fe) in the PVA used, which catalyzed lipid oxidation. Thus, further research on the employment of alternative biopolymers, having a lower price than zein, is required to obtain oxidatively stable fish oil encapsulates by emulsion electrospinning. In addition, an increase in the production rate, which will imply shorter production batches, and the employment of a total inert atmosphere should be also considered in futures studies to reduce oxidation of untrapped fish oil during fibers production.

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FIGURE AND TABLE CAPTIONS

- Figure 1. Properties of electrospinning solutions: a) droplet size distribution after 1 day of production (10.5% PVA, w/w), b) droplet size distribution during storage (10.5% PVA, w/w; WPI), c) droplet size distribution during storage (10.5% PVA, w/w; FPH) and d) viscosity.
- Figure 2. SEM micrographs and fiber diameter distribution of electrospun mats obtained from 10.5% (w/w) PVA-emulsion blends stabilized with WPI and containing different fish oil load: a) 1.5% (w/w) oil, b) 2.25% (w/w) oil and c) 3% (w/w) oil.
- Figure 3. SEM micrographs and fiber and bead diameter distribution of electrospun mats obtained from PVA-emulsion blends stabilized with FPH: a) 10.5% (w/w) PVA-1.5% (w/w) oil, b) 9% (w/w) PVA-2% (w/w) oil and c) 7.5% (w/w) PVA-2.5% (w/w) oil.
- Figure 4. Lipid distribution within the fibers: a) confocal image, b) variation of droplet size distribution.
- Figure 5. Increase of the concentration of secondary oxidation products during storage at 40 °C in a) unprotected fish oil, b) emulsified fish oil, and c) electrospun fibers.
- Table 1. Composition (% , w/w) of PVA-emulsion blends for electrospinning.
- Table 2: Peroxide value during storage at 40 °C for unprotected and emulsified fish oil, fibers and PVA-emulsion blends.

Table 1. Composition (% , w/w) of PVA-emulsion blends for electrospinning

Emulsifier	Fish oil load in emulsion, % (w/w)	Solutions for electrospinning		
		Exp.#	Concentration of PVA, % (w/w)	Concentration of fish oil, % (w/w)
WPI	5.0	1	10.5	1.50
		2	9.0	2.00
		3	7.5	2.50
	7.5	4	10.5	2.25
		5	9.0	3.00
		6	7.5	3.75
	10.0	7	10.5	3.00
		8	9.0	4.00
		9	7.5	5.00
FPH	5.0	10	10.5	1.50
		11	9.0	2.00
		12	7.5	2.50
	7.5	13	10.5	2.25
		14	9.0	3.00
		15	7.5	3.75
	10.0	16	10.5	3.00
		17	9.0	4.00
		18	7.5	5.00

Table 2: Peroxide value during storage at 40 °C for unprotected and emulsified fish oil, fibers and PVA-emulsion blends

PV, meq O ₂ /kg oil								
	Oil	Emulsion	Fibers	¹ Fibers, N ₂	² PVA (13.5%) fibers, N ₂	³ PVA (10.5%)-EDTA emulsion blend	⁴ PVA (10.5%) + EDTA fibers, N ₂	⁵ PVA (10.5%)- emulsion blend
Day 0	0.4±0.1 ^a	0.2±0.1 ^a	54.6±9.3 ^{b,c}	42.5±3.2 ^b	64.5±1.1 ^c	14.7±2.6 ^a	43.9±7.8 ^b	16.1±2.2 ^a
Day 4	11.9±0.2 ^{a,b}	2.2±0.1 ^a	393.7±24.7 ^c	304.4±11.0 ^d	-	42.1±1.9 ^{a,b}	-	48.4±0.8 ^b
Day 9	18.3±0.1 ^a	14.9±0.2 ^a	175.1±8.9 ^b	221.0±18.2 ^c	-	50.5±1.1 ^a	-	144.4±9.7 ^b
Day 14	14.7±0.6 ^a	17.7±2.6 ^a	94.9±20.2 ^{a,b}	141.7±51.0 ^{b,c}	-	61.2±1.1 ^{a,b}	-	208.6±0.1 ^c

¹ Fibers produced from 10.5% (w/w) PVA-emulsion blend under nitrogen atmosphere.

² Fibers produced from 13.5% (w/w) PVA-emulsion blend under nitrogen atmosphere.

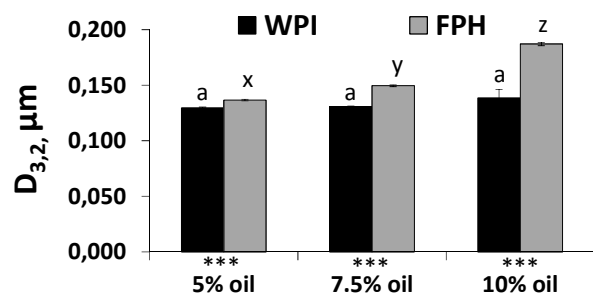
³ 10.5% (w/w) PVA-emulsion blend containing 100 ppm of EDTA in the parent 5% (w/w) fish oil-in-water emulsion stabilized with WPI.

⁴ Fibers produced from the PVA-emulsion blend described in ³ under nitrogen atmosphere.

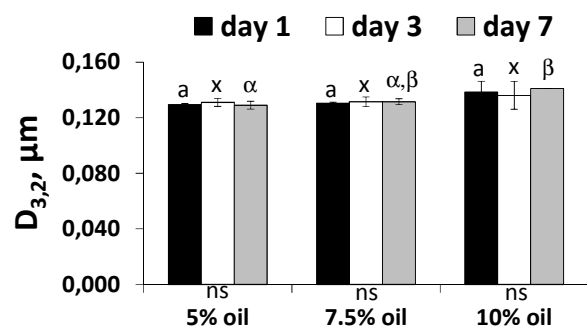
⁵ 10.5% (w/w) PVA-emulsion blend produced with PVA donated by Kuraray Europe GmbH.

Values within a row with different superscript letters indicate significant differences (p < 0.05)

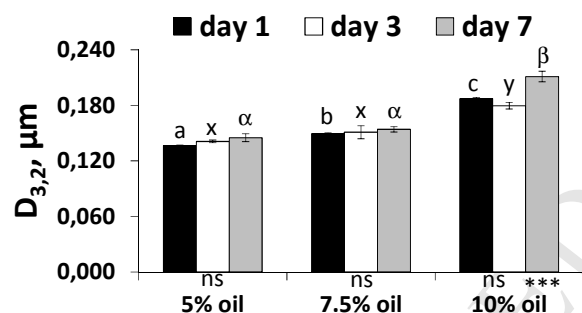
(A)



(B)



(C)



(D)

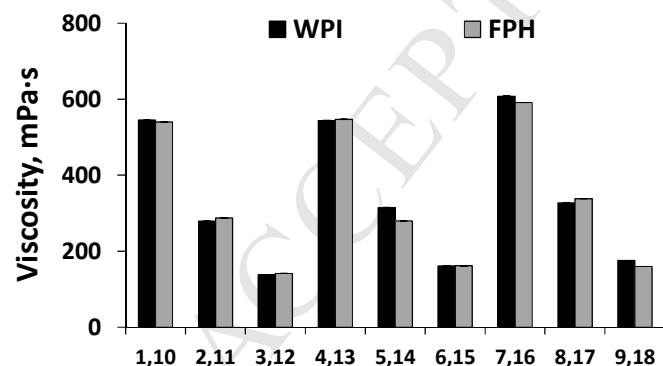


Figure 1. Properties of electrospinning solutions: a) droplet size distribution after 1 day of production (10.5% PVA, w/w), b) droplet size distribution during storage (10.5% PVA, w/w; WPI), c) droplet size distribution during storage (10.5% PVA, w/w; FPH) and d) viscosity.

In Fig. 1a, stars indicate significant differences between WPI and FPH samples for the individual oil concentrations. Letter “a” indicates not significant differences among WPI samples, whereas letters “x”, “y” and “z” indicate significant differences among FPH samples ($p < 0.05$).

In Fig. 1b-c, stars indicate significant differences among samples at day 1, 3 and 7 for the individual oil concentrations (ns: not significantly different). Letters “a, b and c”, “x and y” and Greek letters “α and β” indicate significant differences among samples at day 1, 3 and 7, respectively ($p < 0.05$).

In Fig. 1d, numbers correspond to experiment numbers.

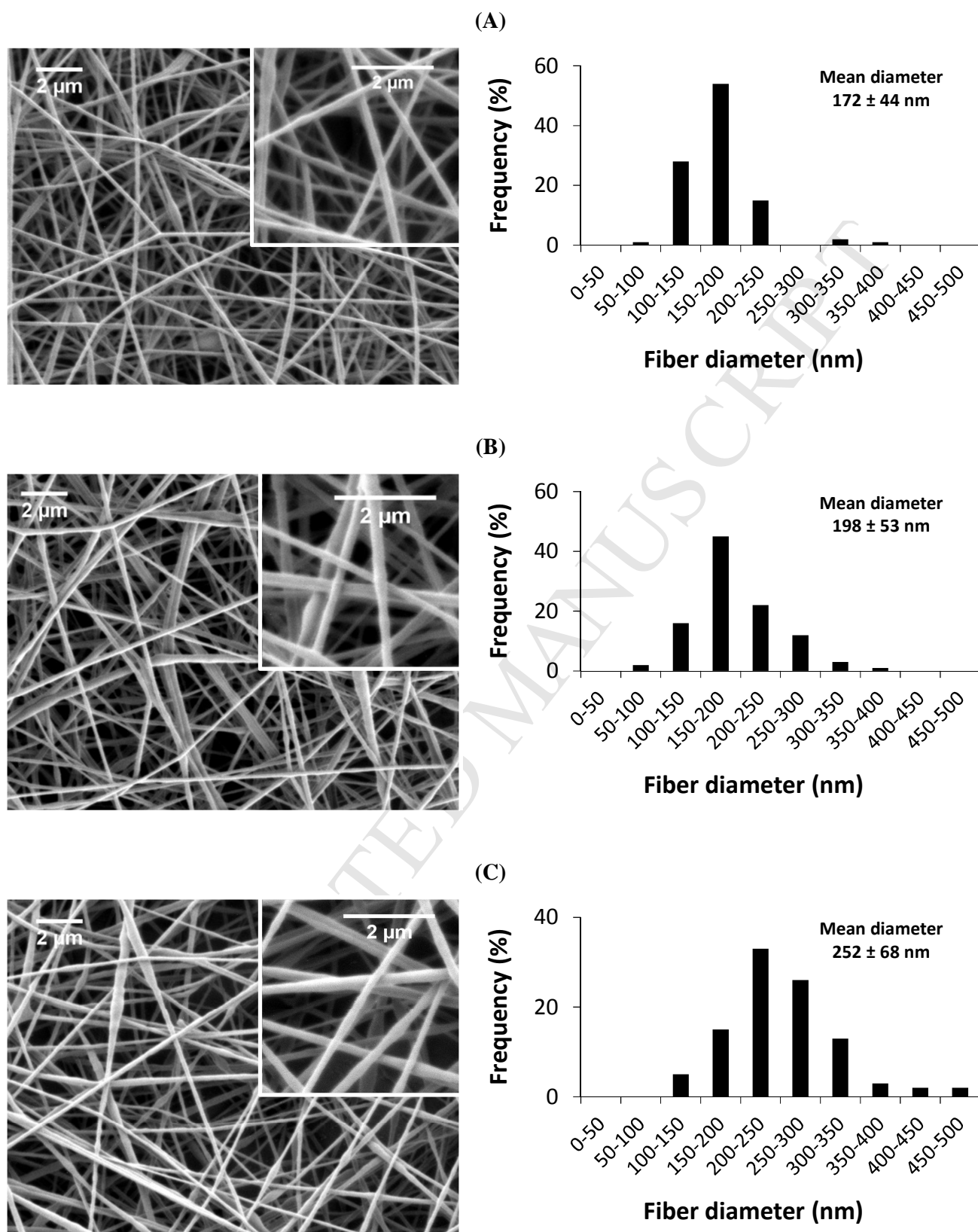


Figure 2. SEM micrographs and fiber diameter distribution of electrospun mats obtained from 10.5% (w/w) PVA-emulsion blends stabilized with WPI and containing different fish oil load: a) 1.5% (w/w) oil, b) 2.25% (w/w) oil and c) 3% (w/w) oil.

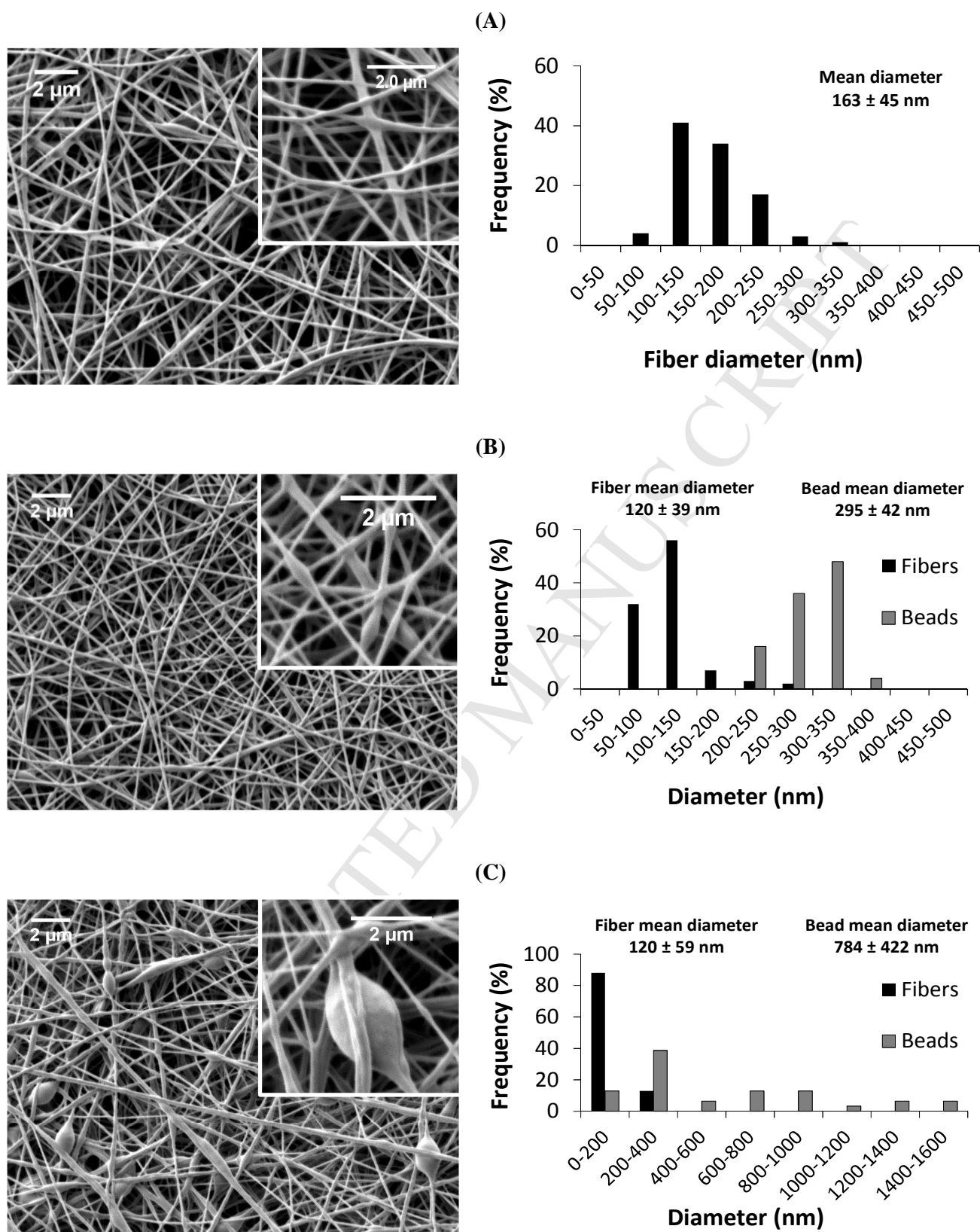
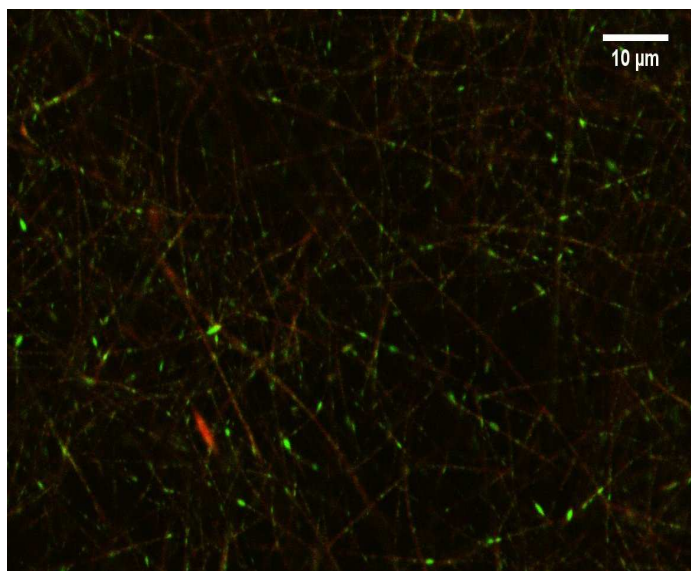


Figure 3. SEM micrographs and fiber and bead diameter distribution of electrospun mats obtained from PVA-emulsion blends stabilized with FPH: a) 10.5% (w/w) PVA-1.5% (w/w) oil, b) 9% (w/w) PVA-2% (w/w) oil and c) 7.5% (w/w) PVA-2.5% (w/w) oil.

(A)



(B)

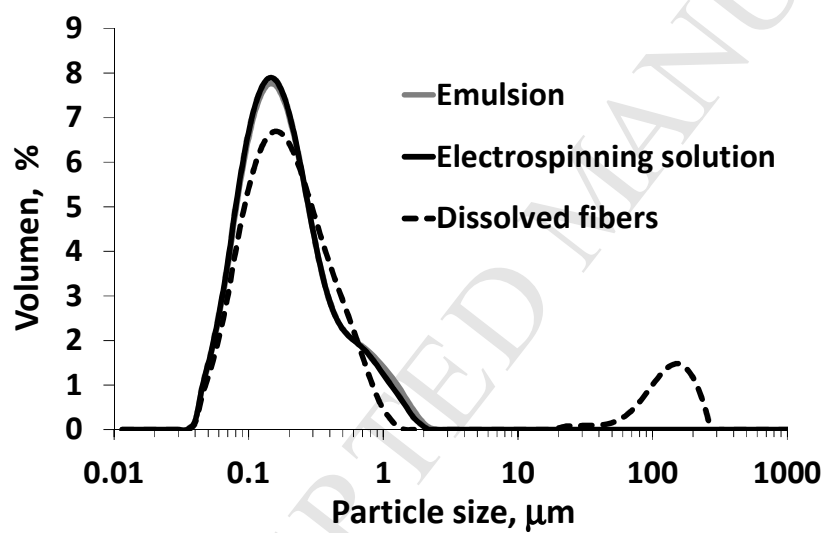


Figure 4. Lipid distribution within the fibers: a) confocal image, b) variation of droplet size distribution.

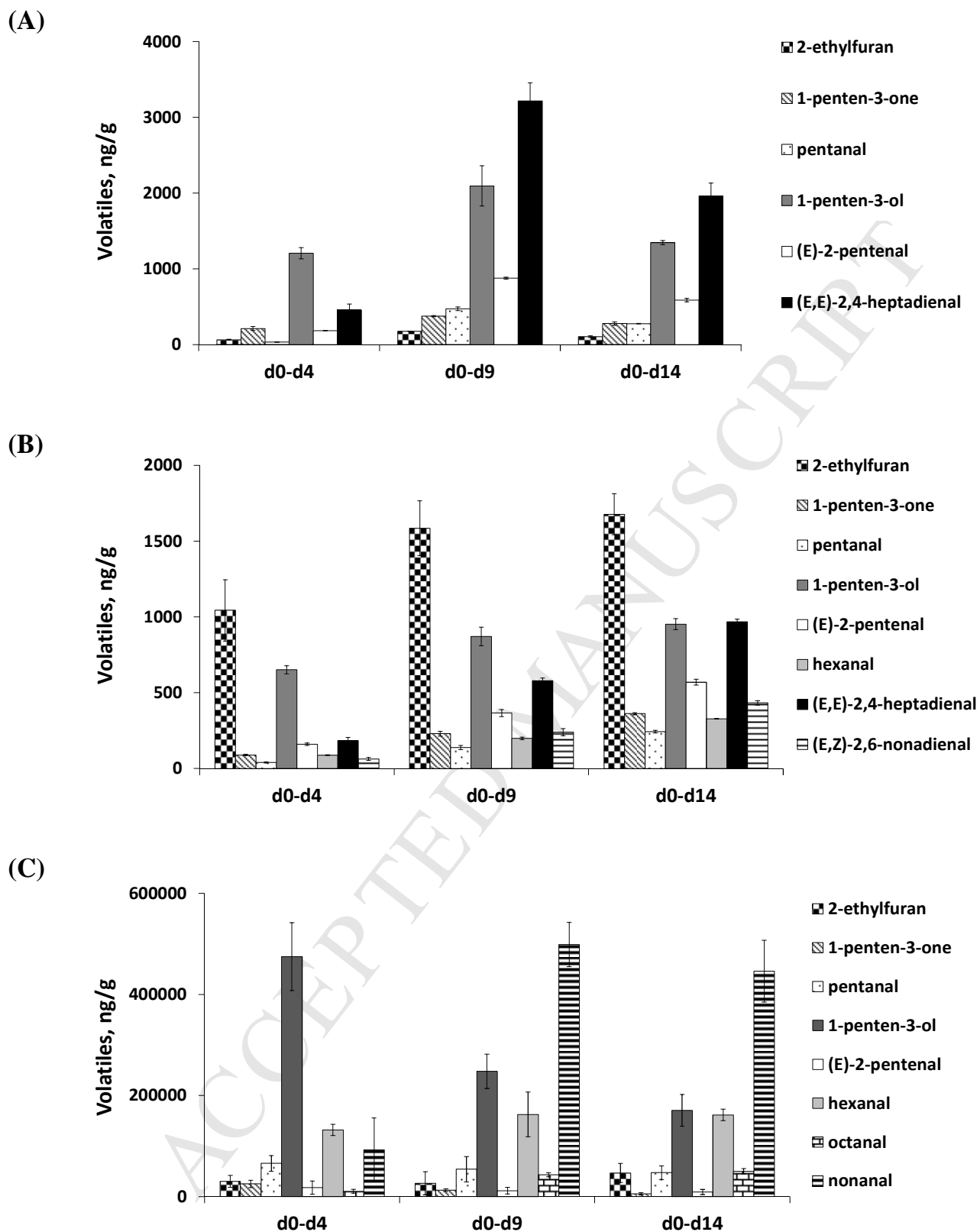


Figure 5. Increase of the concentration of secondary oxidation products during storage at 40 °C in a) unprotected fish oil, b) emulsified fish oil, c) electrospun fibers.

Highlights

- Fibers loaded with fish oil were successfully produced by emulsion electrospinning
- Decreasing PVA concentration (<10.5 wt%) led to spindle-like and spherical beads
- Increasing oil load has a minor influence on fiber morphology
- Most of fish oil was randomly distributed inside the fibers as small droplets
- Electrospun nanofibers presented a poor oxidatively stability